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Dose-Rate Effects of Ethylene Oxide Exposure on Developmental Toxicity

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In risk assessment, evaluating a health effect at a duration of exposure that is untested involves assuming that equivalent multiples of concentration (C) and duration (T) of exposure have the same effect. The limitations of this approach (attributed to F. Haber, Zur Geschichte des Gaskrieges [On the history of gas warfare], in *Funf Vortrage aus den Jahren 1920–1923* [*Five lectures from the years 1920–1923*], 1924, Springer, Berlin, pp. 76–92), have been noted in several studies. The study presented in this paper was designed to specifically look at dose-rate (C × T) effects, and it forms an ideal case study to implement statistical models and to examine the statistical issues in risk assessment. Pregnant female C57BL/6J mice were exposed, on gestational day 7, to ethylene oxide (EtO) via inhalation for 1.5, 3, or 6 h at exposures that result in C × T multiples of 2100 or 2700 ppm-h. EtO was selected because of its short half-life, documented developmental toxicity, and relevance to exposures that occur in occupational settings. Concurrent experiments were run with animals exposed to air for similar periods. Statistical analysis using models developed to assess dose-rate effects revealed significant effects with respect to fetal death and resorptions, malformations, crown-to-rump length, and fetal weight. Animals exposed to short, high exposures of EtO on day 7 of gestation were found to have more adverse effects than animals exposed to the same C × T multiple but at longer, lower exposures. The implication for risk assessment is that applying Haber's Law could potentially lead to an underestimation of risk at a shorter duration of exposure and an overestimation of risk at a longer duration of exposure. Further research, toxicological and statistical, are required to understand the mechanism of the dose-rate effects, and how to incorporate the mechanistic information into the risk assessment decision process.

Key Words: ethylene oxide (EtO); inhalation exposure; developmental toxicity; Haber's Law; mouse.

For risk assessment purposes, the extrapolation of responses from one duration of exposure to another has commonly relied on the use of an approach attributed to Haber (1924), which assumes that the equivalent multiples of concentration (or dose), C, and duration (or time), T, of exposure have the same effect on response. This C × T dose-rate approach (referred to as Haber's Law) has been used by regulatory agencies to estimate the effect of an untested dose-duration combination, using the response of a tested dose-duration combination at the same C × T multiple (Kimmel, 1995). For example, data from a study in which animals were exposed to 300 ppm for 6 h/day would be adjusted for continuous exposure by multiplying 300 by 6/24 resulting in 75 ppm for a 24-h exposure. The assumption is that the response seen after 300 ppm for 6 h/day would be of the same type and magnitude as that seen after 75 ppm for 24 h/day. In fact, Haber (1924) proposed this relationship in the context of evaluating very short-term effects at high concentrations of mustard gas used in warfare, and did not suggest that it be used for extrapolating to much longer exposure durations. Thus, use of Haber's Law for the extrapolation to a longer duration of exposure is an over-simplification of the C × T relationship.

Several studies have been published that show the limitations of using Haber's C × T assumption on both quantitative and qualitative bases. For example, Yoshimura *et al.* (1992) showed that the same chemical led to different types of neurotoxic end points when administered to dogs and rats at high doses for short periods as compared to low doses for longer periods. Crofton *et al.* (1996) found that the behavioral effects of acrylamide depended on both the level and duration of exposure, but the recovery of behavioral function was independent of the duration of dosing. Bushnell (1997) and Crofton and Zhao (1997) showed that the concentration of inhaled trichloroethylene had a greater effect on signal detection behavior and ototoxicity in rats than did duration of exposure. The importance of peak exposure for ozone and sulfur dioxide has been pointed out by several investigators (Hogsett *et al.*, 1985; Lefohn and Jones, 1986; Musselman *et al.*, 1983, 1986; Rappaport, 1991), and has led to exposure indices that placed greater weight on higher concentrations of ozone than on lower levels.

Responses to exposures during development (prenatal or

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postnatal) depend not only on concentration and duration of exposure, but also on the constantly changing molecular and cellular processes that contribute to changing sensitivity. Thus, exposures during development would not be expected to follow Haber's Law except, perhaps, over a relatively short duration of exposure. The present study was designed to test whether this is in fact true by exposing mice on gestation day (GD) 7 to ethylene oxide (EtO) for a short time. EtO was selected because of its short half-life, demonstrated developmental toxicity, and relevance to regulatory science. The primary occupational exposures are to health care workers employed in sterilization areas, usually in hospital settings. OSHA recently estimated that approximately 100,000 health care technicians might be exposed to ethylene oxide in the workplace. These exposures are typically brief, concentrated bursts that occur when the door of a sterilizing machine is opened (Sun, 1986; U.S. HHS, 1994). It has been estimated that at a typical sterilization facility, the 8-h time-weighted exposure levels are generally below 36 mg/m^3, but that short term exposures of 100 mg/m^3 are common, with occasional peak exposures of 1800 mg/m^3 (WHO, 1985).

As indicated above, several investigators have studied the developmental effects of EtO. Depending on the timing and concentration used, exposure of pregnant animals to EtO has resulted in lowered fetal weight, pre- and post-implantation loss, and skeletal malformations (Generoso *et al.*, 1986, 1987; LaBorde and Kimmel, 1980; Polifka *et al.*, 1996; Rutledge and Generoso, 1989; Saillenfait *et al.*, 1996; Snellings *et al.*, 1982, 1984). In humans, a retrospective study of 4856 dental assistants exposed to EtO during pregnancy showed that exposure was associated with an increased risk for spontaneous abortion, with an estimated relative risk of 2.5 after adjusting for age and other factors (Rowland *et al.*, 1996).

There have been several studies on the dose-rate effects of EtO. Generoso *et al.* (1986) evaluated the dominant lethal effects of EtO in exposed mice. Short, high exposures (1200 ppm × 1.5 hours) showed increased frequency of dominant-lethal mutations over long, low exposures (300 ppm × 6 hours). Sega *et al.* (1988) found that DNA breakage and unscheduled DNA synthesis (UDS) in early spermatids increased by a factor of 3, going from a low exposure of 450 ppm × 4 h to 1800 ppm × 1 h. Sega *et al.* (1991) also showed that, as exposure rate increased from 75 ppm × 4 h to 300 ppm × 1 h, ETO binding to developing sperm, sperm DNA, testis DNA, and hemoglobin also increased.

The present study was designed to look specifically at the dose-rate effects of EtO when a short-term exposure occurred at a specific time in development that could result in clearly defined developmental effects. Several preliminary studies using various concentrations of EtO over varying periods from GD 0–14 were conducted before focusing on GD 7. The adequacy of Haber's Law for developmental toxicity was then evaluated with GD 7 exposure, and several models were developed to fit the data and to explore the applicability of Haber's Law. The present study also goes beyond the previous limited dose-rate studies in that it forms an ideal case study with which to examine statistical issues in risk assessment for dose-rate-studies that have not been previously considered.

TABLE 1
Targeted Experimental Design

Duration (h)	EtO concentration (ppm)	C × T (ppm/h)
1.5	0	0
3.0	0	0
6.0	0	0
1.5	1400	2100
3.0	700	2100
6.0	350	2100
1.5	1800	2700
3.0	900	2700
6.0	450	2700

MATERIALS AND METHODS

Animals. Laboratory procedures were approved by the Harvard Medical Area Standing Committee on animals. C57 BL/6J black mice (Jackson Laboratory, Bar Harbor, ME) were housed in a conventional animal room under controlled conditions (12h:12h light/dark cycle, with lights on at 7:00 A.M). Male mice were housed individually, while females were housed in cages of 3 to 4 animals. The identities of the female animals were recorded using an electronic tracking system. Each female mouse was anesthetized with halothane at 2 months of age to implant a transponder under the skin on the back of the neck.

One to 3 females (>4 months of age) were mated with a single male in the afternoon and checked for the presence of vaginal plugs the following morning (plug date, gestation day 0, GD 0). Males were not bred on consecutive nights. A minimum of 3 weeks elapsed before females without plugs were mated again. Females with a plug were housed separately from those without plugs. After stratification by body weight gain between GD 0 and GD 4–5, animals were assigned randomly to air- or EtO-exposed groups.

Experimental design. Females with a vaginal plug on GD 0 were exposed on GD 7 to a C × T multiple of 0 ppm-h, 2100 ppm-h, or 2700 ppm-h. The gestational day and C × T multiples were selected based on the results of a pilot study and design guidelines developed by Scharfstein and Williams (1994). In the targeted experimental design (Table 1) an equal number of mice were randomized to each of the 9 exposure groups, with air exposures run concurrently with EtO exposures. For planning purposes, we aimed to achieve 18 pregnant animals at each C × T combination. With an anticipated pregnancy rate of 50% out of the animals with plugs, we aimed to expose 36 plugged animals at each C × T combination. In the experiments, between 30 and 76 plugged animals were exposed at the target C × T combinations (Table 2). Two duration periods of 1.75 and 2 h for the 2700 multiple along with air-exposed animals were added to the design because of the high rate of maternal toxicity at the 1800 × 1.5 ppm-h combination. A total of 530 plugged animals were exposed to air or EtO. Among the 441 live mice with plugs, 177 (40%) mice were pregnant.

Exposures. Exposures were conducted in 2 side-by-side chambers with a specially designed closed-loop system. The chambers were fabricated from stainless steel and plexiglass and were approximately 45 × 45 × 60 cm with a pyramid on top to aid in uniform equilibration of the gases. Each chamber had a volume of approximately 100-L. The air flow rate through each chamber was controlled at approximately 45 L/min (range 40–80 L/min). The time

TABLE 2
Mortality and Pregnancy Rates for 530 Mice with Plugs

Ppm × h	Plugs present, *n*	Deaths, *n* (%)	Live, *n* (%)	Pregnant, *n* (%)
Air				
0 × 1.5	50	0 (0.0)	50 (100.0)	28 (56.0)
0 × 1.75	8	0 (0.0)	8 (100.0)	6 (75.0)
0 × 2	28	1 (3.6)	27 (96.4)	14 (51.9)
0 × 3	38	0 (0.0)	38 (100.0)	19 (50.0)
0 × 6	30	1 (3.3)	29 (96.7)	19 (65.5)
C × T = 2100				
1400 × 1.5	39	3 (7.7)	36 (92.3)	8 (22.2)
700 × 3	41	0 (0.0)	41 (100.0)	22 (53.7)
350 × 6	33	0 (0.0)	33 (100.0)	19 (57.6)
C × T = 2700				
1800 × 1.5	73	41 (56.2)	32 (43.8)	3 (9.4)
1543 × 1.75	23	15 (65.2)	8 (34.8)	1 (12.5)
1350 × 2	76	27 (35.5)	49 (64.5)	7 (14.3)
900 × 3	50	1 (2.0)	49 (98.0)	11 (22.5)
450× 6	41	0 (0.0)	41 (100.0)	20 (48.8)
Total	530	89 (16.8)	441 (83.2)	177 (40.1)

required for equilibration of the chamber to 99% concentration under these operating conditions was 10.2 minutes.

Gas was fed online using a "PID" (Proportional Integral Derivative) system. Chamber concentrations were monitored continuously and compared to the set point. The computer calculated the error signal and made appropriate corrections. Whenever animals were being exposed, measurements were taken automatically, including the concentration of EtO, flow rates, and pressure. Actual concentrations were maintained within plus or minus 5% of desired levels after allowing for the initial startup time of the exposure chamber. The laboratory background levels were also monitored for safety.

Maternal and fetal evaluations. Maternal toxicity was evaluated 30 min after exposure to detect short term effects and again 24 h later for more persistent effects. The indicators of short-term toxicity were behavioral and weight changes. The behavioral changes assessed by an examiner, blind to the treatment, were fur appearance (piloerection or soiled versus normal), movement (little or none vs. normal), arousal based on snapping of fingers (no flinching [depressed] versus flinching [normal]), eyes (crusty versus normal), and breathing (labored versus normal). The animals were weighed on GD 0, GD 4/5, immediately prior to exposure, and daily after exposure at regular intervals until GD 18.

Experimental animals were sacrificed with excessive ether anesthesia on GD 18 of gestation. The uterus was removed, opened and the positions of each pup and resorption site in the uterus were recorded. After determining viability, each live pup was weighed on a calibrated Mettler #35 balance. The crown-to-rump length was determined with a hand-held Fowler MAX-CAL electronic digital caliper. An external examination of each pup was carried out under a dissecting microscope. The specific anomalies sought included, among others, anopthalmia/micropthalmia, anotia/microtia, hydrocephalus, cleft lip, abdominal wall defect, polydactyly, syndactyly, spinal bifida, undescended testes, and any type of limb deficiency. The following structures were examined: brain/cranium, ear, eye, nose, jaw, tongue, digits, limbs, spinal cord, tail, heart, lung, intestines, kidney, and uterus/testes. An incision in the lower abdomen was made to examine internal reproductive organs and to assign sex. The patency of the anus, presence of the spleen and 2 kidneys, and the lack of any defect in the diaphragm were confirmed by inspection. The chest was opened and the contents were examined for absence of either lung or major abnormalities of the great arteries of the heart. After evisceration and removal of the skin, the carcass, including the head, was fixed in 95% alcohol, skeletons were stained with alcian blue and alizarin red S, and soft tissue was cleared in 1% potassium hydroxide (McLeod, 1980). Information on the skeletal effects will be reported in a subsequent paper.

Statistical analysis. Three dose-response models were fitted to each of the maternal and developmental end points. These included Haber's Law model (i.e., including only the C × T multiple) and 2 models that allowed for deviations from Haber's Law. These 3 models are described in more detail below for the binary end points (maternal death, clinical signs, fetal death, malformation). The analogous 3 models can be fit for the continuous measurements using mean response (maternal body weight change, fetal weight, crown-to-rump length). Generalized estimating equations (Liang and Zeger, 1986) were used to adjust for correlation between littermates.

Standard dose-response models for developmental toxicity are a function of 'dose' only and do not account for varied duration of exposure. Under Haber's Law, the important dose metric is assumed to be the dose-duration multiple, i.e., C × T, so that the 'standard' dose-response model would be rewritten as

$$\text{logit}(p) = \alpha + \beta * C \times T, \quad (1)$$

where p is the probability of an adverse event (e.g., death), α is the intercept on the logit scale, and β is the log-odds associated with exposure to the specified C × T multiple. This model assumes that concentrations and times of duration that provide the same C × T multiple have the same predicted response.

Two deviations from Haber's model, described by Scharfstein and Williams (1994) to address more complex dose-rate patterns of effects, were fit to the data. The first one assumes that concentrations and times of duration that provide the same C × T multiple does not necessarily have the same predicted response. To allow for this in the model, a term is added for the duration of exposure in addition to the C × T multiple. This model is of the form

$$\text{logit}(p) = \alpha + \beta * C \times T + \gamma * T, \quad (2)$$

where γ is the parameter corresponding to the exposure duration. The second generalization further assumes that air-exposed animals display a similar level of effect regardless of exposure duration. To model this, an indicator function δ is included as follows:

$$\text{logit}(p) = \alpha + \beta * C \times T + \gamma * \delta * T, \quad (3)$$

where $\delta = 1$ if $C > 0$ and 0 otherwise. To assess whether Haber's model was appropriate, we fitted models 2 and 3 and then conducted a test of significance for the coefficient associated with duration (γ).

For each of the models specified above, the effective C × T contour, as defined by Scharfstein and Williams (1994), is generated using the parameter estimates of α, β, and γ. This contour provides estimates of C × T that correspond to a specified estimated excess risk (5%) above background (defined as C = 0 and T→0). For example, the effective C × T contour computed for Equation 2, using the estimated parameters computed for Model 2 is defined as the set of (C,T)-values that satisfy:

$$\hat{\beta} * C \times T + \hat{\gamma} * T = \text{logit}(0.05). \quad (4)$$

This contour provides a range of estimated C × T values that correspond to a specified excess risk. Therefore, it is possible for the combinations of concentration and duration that result in a specified excess risk to be values not used in the experimental design.

RESULTS

Pilot Study Results

Identification of the C × T multiples to be used in this study was more complicated than in a standard dose-response experiment because of the interaction between the concentration and duration of exposure. To determine the maximum C × T multiple that produced minimal maternal toxicity and the ideal gestational timing of exposure, two pilot studies were conducted: (1) to establish the concentration-duration range of maternal toxicity, and (2) to establish that developmental abnormalities occur within the C × T range identified and to identify sensitive times of exposure within the gestation cycle.

Based on several experiments, 2700 ppm-h was identified as the highest multiple for the main study. These experiments focused primarily on 1-h exposures, since the most toxicity was expected at the short, high exposures. After several experiments involving different 1-h exposures, it became clear that a fairly high rate of maternal toxicity would have to be accepted at the 1-h exposures to see significant fetal effects. As a result, the 1.5-h exposure time was selected as the shortest duration with 2700 ppm-h as the highest C × T multiple. For example, the maternal death rate at 1800 × 1.5 ppm-h was considerably lower (23%) than the rate among animals exposed to 2700 ppm for 1 h (90%). The second multiple used in our main experiment (2100 ppm-h) was chosen because pilot data had suggested that exposures much lower than this were unlikely to produce fetal effects. Furthermore, the optimal design considerations of Scharfstein and Williams (1994) suggested that the 2100 multiple would be a good choice.

In the second pilot study, the goal was to determine the most sensitive day for exposure. Six timed-pregnant dams were exposed to 900 ppm for 3 h separately, on each gestational day between 6 and 14 for a total of 54 pregnant mice. In addition, 4 pregnant females on each gestational day (36 total) were exposed to air. Comparing the effects, gestational day 7 was the most sensitive day. Of the 33 implantations detected in the 4 pregnant females exposed on this day, 4 (12%) were resorbed and 4 (12%) were stillborn. In addition, 8 (32%) of the 25 live offspring were malformed. Among the types of malformations observed were micropthalmia, anopthalmia, and hydrocephalus.

On the basis of these pilot study results, the main study was designed to document the extent and types of developmental effects associated with varying levels of both the concentration and duration of exposure on gestational day 7.

Main Study Results

Five hundred thirty female mice with vaginal plugs were exposed to either air or ethylene oxide on GD 7. Table 2 shows the final distribution of animals and the number of maternal animals exposed at each C × T combination. Among the 530 mice with plugs, 89 (16.8%) died (84 of these mice had been exposed to the highest multiple). Among the 441 that survived to sacrifice at GD 18, 177 (40%) were found to be pregnant, with the pregnancy rate ranging from 50% to 75% in the air-exposed animals, from 22% to 58% in the animals exposed at the 2100 multiple and from 9.4% to 49% in the animals exposed at the 2700 multiple. Clearly, there is a dose-related trend in the pregnancy rates with the lower rates observed at the short, high exposures (e.g., 1400 × 1.5 and 1800 × 1.5). In fact, modeling pregnancy rate as a function of C × T in addition to duration (T) showed that the pregnancy rate varied significantly across the concentration-duration combinations (Model 2), the estimated γ and β were significant with $p = 0.0001$). This is probably due to early resorptions that were not detected rather than to reduced fertility. This suggests that the results from the analysis of resorptions discussed subsequently may underestimate the true rate of resorptions, especially in the ppm-h combinations where the pregnancy rates observed were much lower than the air-exposed rates.

The majority of the maternal deaths occurred at a short duration of exposure within the 2700 multiple. It is clear that the observed maternal death rate varied depending upon the C × T combination (Table 2, Fig. 1a). The predicted response surface plots from fitting Model 1, i.e., Haber's model, which assumes that the C × T multiple is the important dose metric, and Model 2, which includes a term for the duration of exposure in addition to one for the C × T multiple, are given in Figures 1b and 1c. Results from fitting Model 3 were very similar to Model 2 for all endpoints and therefore were omitted in subsequent figures and discussion. The most notable feature is that long, low exposures to EtO (e.g., 450 × 6) did not lead to deaths, unlike what would be predicted from the Haber's Law model. This can also be seen from Table 2, for example, within the 2700 multiple where the observed death rate ranges from 0% at the 450 × 6 ppm-h combination to 65.2% at the 1543 × 1.75 ppm-h combination. This departure from Haber's model is quantitatively reflected by the significance of the duration parameter estimate for maternal mortality ($p = 0.0001$).

To interpret these predicted response surfaces, consider Figure 1b, constructed under the assumption of Haber's Law. Imagine slicing the dose-response surface at a fixed level of response. This yields a set of points with each point on the C × T contour corresponding to the same C × T multiple (i.e., a fixed C × T predicts a constant response). Model 2 leads to a predicted response surface with a different shape (Fig. 1c). Slicing this dose-response surface at a fixed level of response to generate the effective C × T contour results in a different set of concentrations and duration times. The product of these concentrations and duration times do not all equal the same C × T multiple. For these two models, the contours are shown in Figure 1d. Under Haber's model, a C × T combination of 1774 × 1 ppm-h gave the same predicted probability of maternal death as the 295.7 × 6 ppm-h combination. Under Model 2, however, the predicted probability (p) of maternal

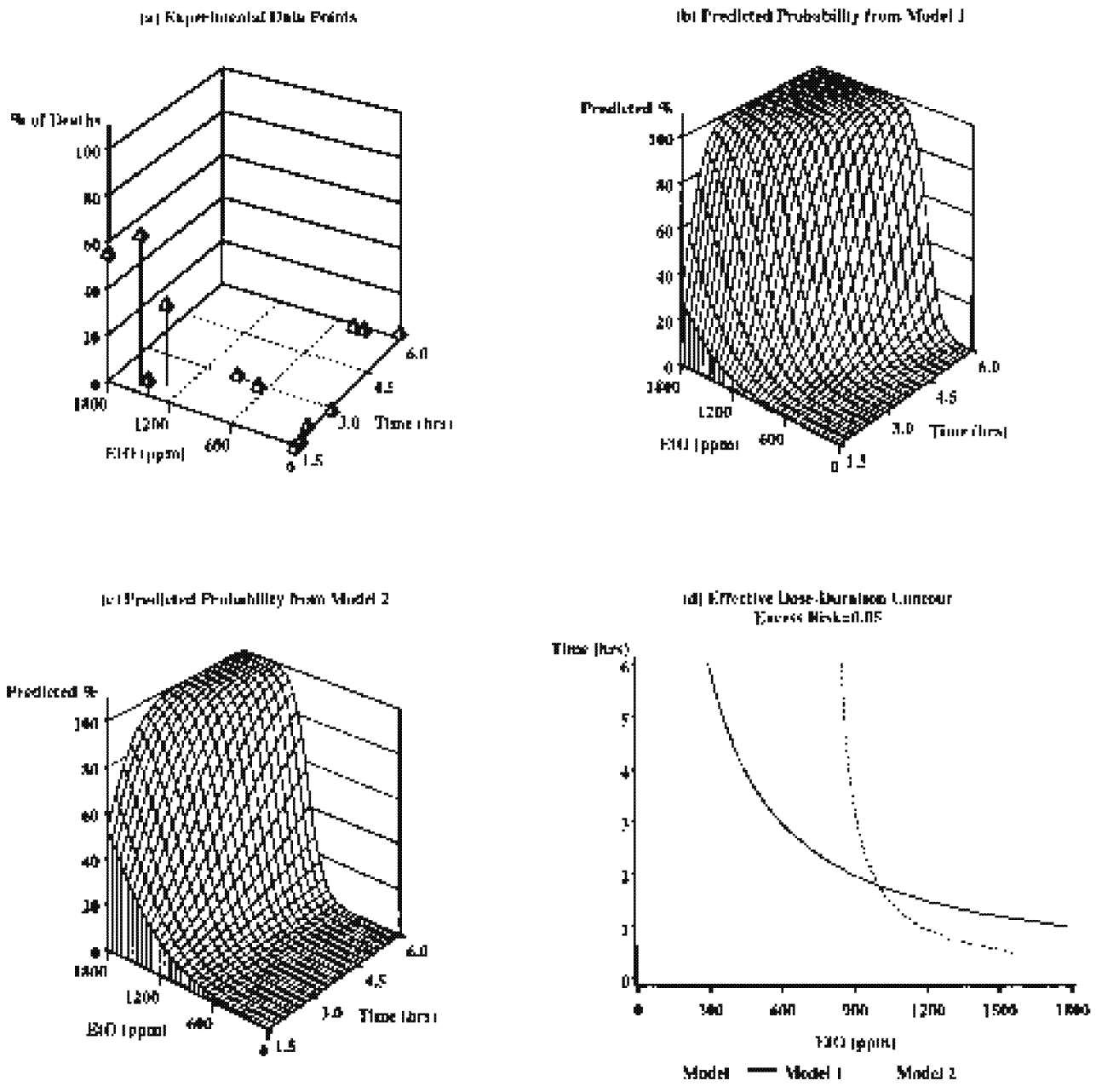

FIG. 1. Maternal mortality from EtO exposure.

death was 0.27 for the 1774 × 1 ppm-h combination and 0.0001 for the 295.7 × 6 ppm-h combination. This is based on the following fitted regression models for Haber's model:

$$\text{logit}(p) = -6.853 + 0.002\ \text{C} \times \text{T}, \quad (5)$$

and Model 2:

$$\text{logit}(p) = -3.049 - 1.615 * T + 0.002 * C \times T, \quad (6)$$

Therefore, applying Haber's model at short duration times would severely underestimate the risk, whereas at longer times it would severely overestimate the risk.

Maternal effects from exposure to EtO are readily apparent from the observed percent body weight loss (Table 3) and the observed clinical signs (Table 4). The percent body weight loss ranges from 0.3 to 1.2% in the mice exposed to air; whereas in the animals exposed to EtO it was significantly higher, ranging from 4.7 to 7.2% in the 2100 multiple and 6.2 to 13.5% in the 2700 multiple. None of the air-exposed animals and up to 59.2% of the EtO-exposed animals exhibited depressed

TABLE 3
Percent Maternal Body Weight Loss1 Day Post Exposure

Ppm × h	Number of mice exposed	Percent loss, mean (SE)
Air		
0 × 1.5	50	1.2 (0.33)
0 × 1.75	8	0.7 (0.53)
0 × 2	28	0.3 (0.45)
0 × 3	38	3.4 (0.88)
0 × 6	30	3.8 (0.30)
C × T = 2100		
1400 × 1.5	39	7.2 (0.90)
700 × 3	41	6.6 (0.38)
350 × 6	33	4.7 (0.36)
C × T = 2700		
1800 × 1.5	73	13.0 (0.49)
1543 × 1.75	23	13.5 (0.72)
1350 × 2	76	11.4 (0.54)
900 × 3	50	8.8 (0.57)
450 × 6	41	6.2 (0.39)

arousal. The changes in diarrhea and fur appearance are not included in the analysis, because it was difficult to determine which of the 3–4 female mice in a cage had diarrhea, and because fur appearance turned out to be an insensitive indicator of maternal toxicity. In the analysis, each animal was identified as abnormal if it had little or no movement, depressed arousal, crusty eyes, or labored breathing. Among the animals exposed to air, 0 to 12.5% had abnormal clinical signs 30 min after exposure (Fig. 2a); whereas, among the animals exposed to EtO, 53.1 to 100% in the 2100 multiple and 95.1 to 100% in the 2700 multiple had abnormal clinical signs (Table 4). The shape of the predicted response surface under Haber's model (Fig. 2b) and under Model 2 (Fig. 2c) were slightly different. The difference in these two response surfaces were primarily driven by the effects seen at the 2100 multiple, where the percent of animals observed with abnormal clinical signs was much higher at the 1400 × 1.5 ppm-h (100%) than at the 350 × 6 ppm-h combination (53.1%). The modeling results for percent body weight loss and abnormal clinical signs both indicate a significant departure from Haber's Law (p = 0.0001).

Based on the C × T contours for the abnormal clinical signs percentage, the application of Haber's model tends to underestimate the risk at short duration and overestimate the risk at long duration, as was seen previously (Fig. 2d). This can be seen by selecting 2 points on the effective C × T contour that results in 5% excess risk: 447 × 1 and 74.5 × 6 ppm-h. The predicted probability of abnormal clinical signs for the 447 × 1 ppm-h combination was 0.07 under Haber's model and 0.18 under Model 2 resulting in the risk being underestimated. At the longer durations the risk was overestimated with a predicted probability at the 74.5 × 6 ppm-h combination of 0.07 under Haber's model and 0.02 under Model 2.

The fetal effects from exposure to EtO are also readily apparent from the observed fetal death rate, malformation rate and the reduction in growth (Table 5). The observed fetal death rate (proportion of resorptions or stillbirths) ranged from 6 to 13.8% in the air-exposed mice and from 9.2 to 63.6% in the EtO-exposed mice (Fig. 3a, Table 5). Among the live offspring in the EtO exposure groups, 10.8 to 100% were malformed

TABLE 4
Percent Maternal Clinical Signs 30 Minutes/24 Hours Post Exposure

	30 Minutes					24 Hours				
Ppm × h	Abnormal %	Little/no movement %	Depressed arousal %	Crusty eyes %	Labored breathing %	Abnormal %	Little/no movement %	Depressed arousal %	Crusty eyes %	Labored breathing %
Air										
0 × 1.5	2.3	0.0	0.0	2.3	0.0	0.0	0.0	0.0	0.0	0.0
0 × 1.75	12.5	0.0	0.0	12.5	0.0	12.5	0.0	0.0	12.5	0.0
0 × 2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0 × 3	2.6	2.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0 × 6	6.7	6.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C × T = 2100										
1400 × 1.5	100.0	87.2	38.5	20.5	92.3	20.7	10.3	6.9	17.2	3.5
700 × 3	81.6	79.0	13.2	10.5	76.3	5.3	0.0	2.6	2.6	0.0
350 × 6	53.1	34.4	0.0	28.1	46.9	3.1	0.0	0.0	3.1	0.0
C × T = 2700										
1800 × 1.5	100.0	91.8	49.3	27.9	100.0	66.2	58.1	54.9	25.0	55.6
1543 × 1.75	95.7	95.7	17.4	26.1	95.7	72.2	66.7	38.9	16.7	72.2
1350 × 2	100.0	89.5	59.2	19.7	98.7	39.7	32.4	33.8	13.2	35.3
900 × 3	98.0	98.0	42.0	14.0	96.0	24.0	8.0	2.0	8.0	20.0
450 × 6	95.1	73.2	14.6	22.0	85.4	2.4	0.0	0.0	0.0	2.4

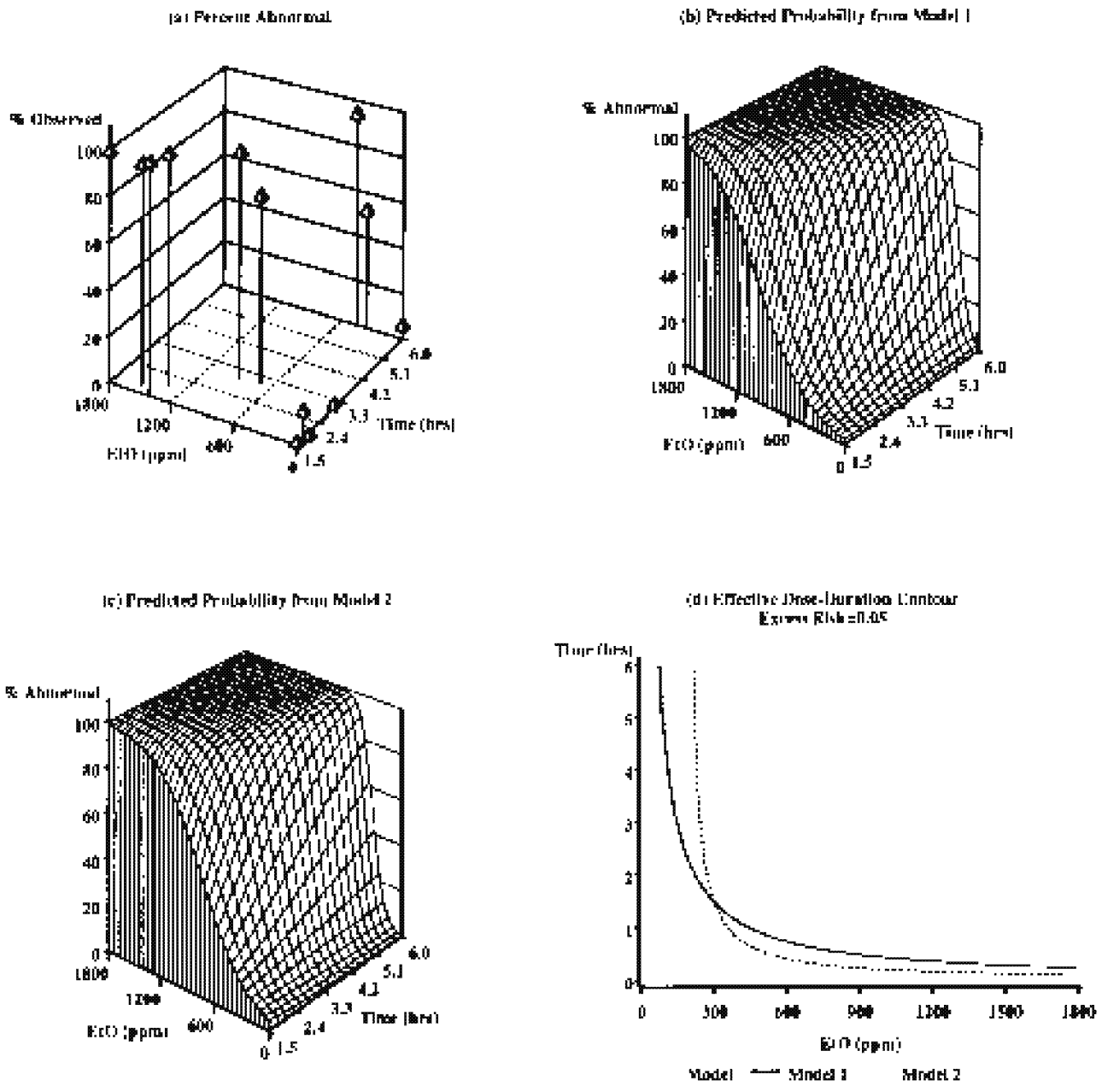

FIG. 2. Percent of mice with abnormal clinical signs 30 min post exposure.

compared to 4 to 10.6% of the air-exposed animals (Fig. 3b, Table 5). An offspring was identified as malformed if any of the structures examined were determined to be abnormal. The different shape of the predicted surface under Haber's model and Model 2 in Figures 3c–3f for fetal death and malformation show significant departures from Haber's Law ($p < 0.0002$). As has been noted, this means that prediction of the frequency of fetal effects based on Haber's Law for exposures of short duration would underestimate risk and for longer duration it would overestimate risk.

Table 6 shows the incidence of specific types of malformations in all of the exposure groups. Microphthalmia and anophthalmia were the malformations that occurred most frequently in all exposure groups. The C57BL/6J strain of mouse has a low background frequency of eye defects (4–11% in this study). There was a significant increase in the frequency of eye defects in the EtO exposed groups in both of the dose-duration multiples (14 to 38% in the 2100 multiple and 30 to 100% in the 2700 multiple). With greater duration, the incidence tended to decrease, so that in the 6-hour duration groups in both multiples the incidence was similar to that in the control groups (14% at 350 × 6 ppm-h and 11% at 450 × 6 ppm-h).

TABLE 5
Pregnancy Outcomes

Ppm × h	Preg *n*	Implant *n*	Resorp *n* (%)	Stillborn *n* (%)	Live *n* (%)	Malf *n* (%)	Fetal Wt (g) mean (SE)	Crown to rump length (mm) mean (SE)
Air								
0 × 1.5	28	203	28 (13.8)	0 (0.0)	175 (86.2)	13 (7.4)	0.92 (0.012)	19.22 (0.125)
0 × 1.75	6	50	3 (6.0)	0 (0.0)	47 (94.0)	5 (10.6)	0.97 (0.011)	20.03 (0.115)
0 × 2	14	95	11 (11.6)	1 (1.1)	83 (87.4)	4 (4.8)	0.99 (0.014)	20.70 (0.136)
0 × 3	19	141	15 (10.6)	1 (0.7)	125 (88.7)	5 (4.0)	0.93 (0.011)	19.71 (0.122)
0 × 6	19	150	14 (9.3)	0 (0.0)	136 (90.7)	14 (10.3)	0.99 (0.010)	19.52 (0.124)
C × T = 2100								
1400 × 1.5	8	62	24 (38.7)	17 (27.4)	21 (33.9)	7 (33.3)	0.72 (0.048)	16.89 (0.671)
700 × 3	22	169	27 (16.0)	3 (1.8)	139 (82.2)	56 (40.3)	0.88 (0.012)	19.24 (0.148)
350 × 6	19	152	13 (8.6)	1 (0.7)	138 (90.8)	20 (14.5)	0.97 (0.010)	19.90 (0.123)
C × T = 2700								
1800 × 1.5	3	22	14 (63.6)	0 (0.0)	8 (36.4)	7 (87.5)	0.70 (0.064)	16.66 (0.739)
1543 × 1.75	1	7	1 (14.3)	0 (0.0)	6 (85.7)	6 (100.0)	0.76 (0.030)	17.83 (0.356)
1350 × 2	7	20	9 (45.0)	1 (5.0)	10 (50.0)	3 (30.0)	0.86 (0.103)	18.74 (1.082)
900 × 3	11	86	22 (25.6)	5 (5.8)	59 (68.6)	34 (57.6)	0.82 (0.016)	18.42 (0.203)
450 × 6	20	148	28 (18.9)	0 (0.0)	120 (81.1)	13 (10.8)	0.97 (0.010)	19.32 (0.121)

A significant decrease in fetal weight (g) is apparent in the EtO-exposed groups (Table 5), especially in the offspring of the dams exposed at the 2700 multiple. The mean fetal weight ranged from 0.70 to 0.97, which is low compared to the air-exposed animals (means ranging from 0.92 to 0.99). The changes in crown-to-rump length showed a similar pattern (Table 5). Due to a combination of maternal toxicity and fetal deaths, there were fewer live pups at the high exposure, short duration combinations relative to the low exposure, longer duration combinations and air exposures. Even so, there was a highly significant departure from Haber's model for both growth measures ($p = 0.0001$).

DISCUSSION

The results of this study provide convincing evidence that Haber's Law is an oversimplification of the relationship between dose, duration, and response for even very short-term exposures to EtO. The potential error in using Haber's Law to extrapolate from one duration of exposure to another is that it could possibly lead to an underestimation of risk at shorter duration of exposure and an overestimation of risk at longer duration of exposure. Such a consequence is shown clearly in this paper for both maternal and developmental toxicity endpoints.

This study confirms that airborne exposure to high EtO exposure levels of short duration, versus lower exposure levels of longer duration can have significant effects on fetal death, malformations, fetal weight, and crown-to-rump length. This relationship is similar to that reported by Generoso *et al.* (1986) and Sega *et al.* (1988, 1991) for EtO effects on various measures of germ cell toxicity, and it may be due to pharmacokinetic differences at various exposure concentrations. Brown *et al.* (1998) have shown that the relationship between external exposure concentration of EtO and internal dose is not linear at exposures above 200 ppm for 4 h. This significant departure from linearity is likely due to glutathione (GSH) depletion, the major conjugation elimination pathway for EtO. GSH, GSH-related enzymes, and antioxidant enzymes are present in the adult as well as in the conceptus and in the developing placenta (Tiboni *et al.*, 1997). Brown *et al.* (1998) showed that GSH levels were reduced in liver and lung of adult male B6C3F1 mice after EtO exposures as low as 100 ppm for 4 h, with reductions in kidney and testis at 300 and 400 ppm as well. No studies have reported GSH levels in maternal animals or embryos after EtO exposure during pregnancy; but it is likely that they would be affected, since studies on several other agents, including valproic acid (Andrews *et al.*, 1998), acetaminophen (McLellan *et al.*, 1998), phenytoin and benzo [a] pyrene (Win and Wells, 1997), and thalidomide (Hansen *et al.*, 1998) have reported reductions in GSH levels in maternal animals, yolk sac, placenta, and embryos.

In terms of outcome, the findings in this paper are consistent with those observed in previous EtO experiments (fetal death, growth reduction, and malformation) and provide additional information on very short exposures at higher durations to what has been reported in the literature on repeated brief exposures. For example, LaBorde and Kimmel (1980) exposed pregnant CRL: COBS CD1 (ICR) mice intravenously to 0, 75, or 150 mg/kg for 3 consecutive days during 4 gestational-day periods (4–6, 6–8, 8–10, and 10–12). At 150 mg/kg, reductions in fetal body weight were observed for all 4 time periods, resorptions were increased with dosing on days 8–10 and 10–12, and

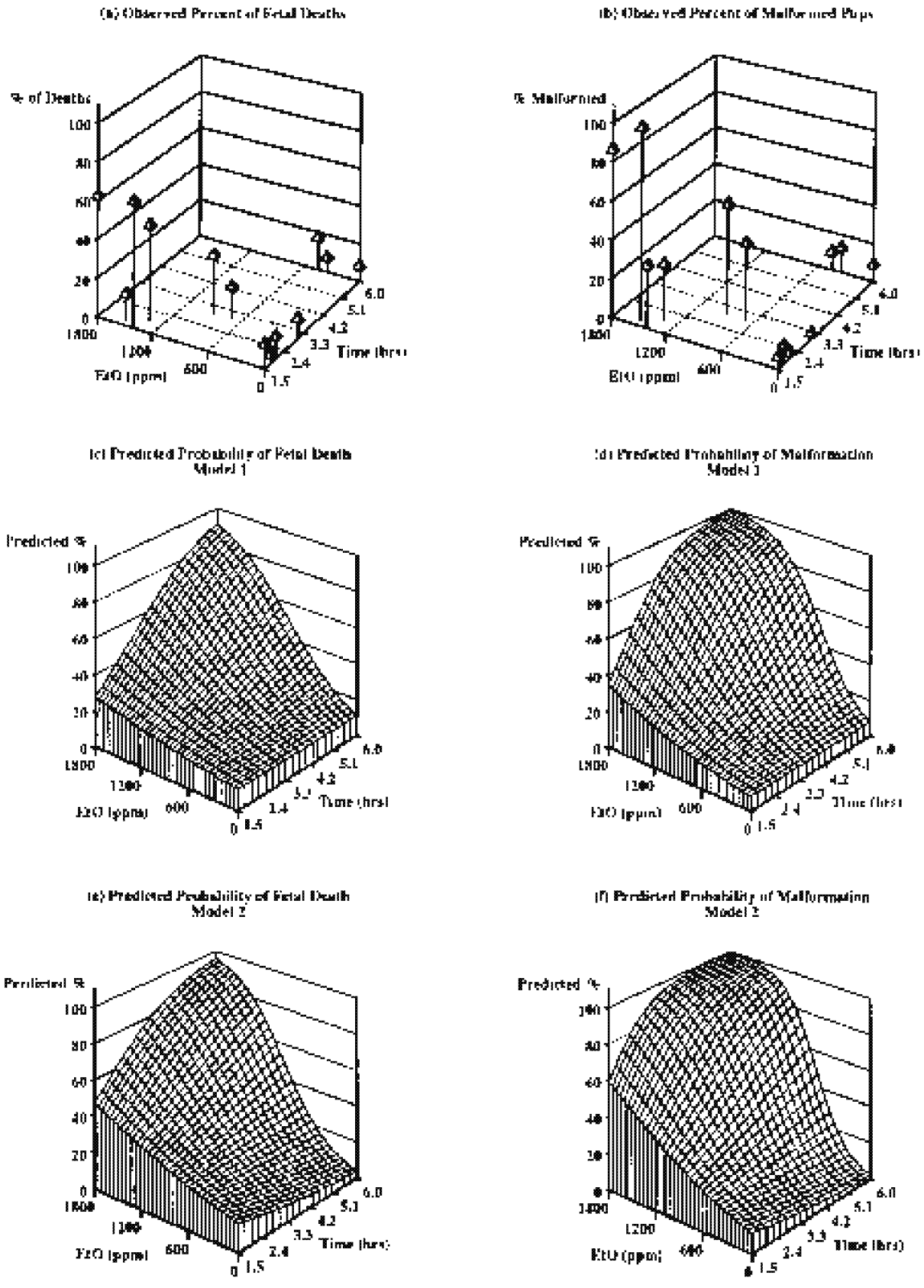

FIG. 3. Fetal death and malformation.

TABLE 6
Observed Malformations at each C × T Combination

	0 × 1.5	0 × 1.75	0 × 2	0 × 3	0 × 6	1400 × 1.5	700 × 3	350 × 6	1800 × 1.5	1543 × 1.75	1350 × 2	900 × 3	450 × 6
Number offspring	175	47	83	125	136	21	139	138	8	6	10	59	120
Number litters	28	6	14	19	19	8	22	19	3	1	7	11	20
Total Number (%) Affected Litters	6 (21%)	3 (50%)	3 (21%)	4 (21%)	6 (32%)	3 (38%)	15 (68%)	8 (42%)	1 (33%)	1 (100%)	2 (29%)	9 (82%)	10 (50%)
Total Number Eye Defects (%)	13 (7%)	5 (11%)	4 (5%)	5 (4%)	12 (9%)	7 (33%)	53 (38%)	20 (14%)	7 (88%)	6 (100%)	3 (30%)	34 (58%)	13 (11%)
Specific Malformations[a]													
Agnathia							4 (2)	1 (1)	1 (1)		1 (1)		1 (1)
Anophthalmia				1 (1)		2 (2)	23 (8)	1 (1)	4 (1)	3 (1)	2 (2)	11 (6)	6 (6)
Anotia						1 (1)							
Exencephaly						1 (1)	4 (3)				1 (1)	1 (1)	1 (1)
Microphthalmia	13 (6)	5 (3)	4 (3)	4 (4)	2 (6)	7 (3)	41 (15)	19 (8)	4 (1)	6 (1)	2 (1)	25 (9)	9 (9)
Missing Lung Lobe								1 (1)					
No Intestines												1 (1)	
No Tongue							1 (1)						
Rudimentary Tongue							1 (1)						
Short Snout												1 (1)	1 (1)
Short Tail							1 (1)						

[a] Entries for eye malformations are the number of offspring affected and in parenthesis the number of litters these offspring came from.

malformations (primarily skeletal changes such as fused cervical and thoracic arches) were seen after dosing on GD 6–8. In contrast, Snellings *et al.* (1982) studied the fetal effects of relatively low-level inhalation exposures of 0, 10, 33, or 100 ppm EtO for 6 h per day to Fischer 344 rats on gestational days 6 through 15. The only effect detected was lowered fetal body weights in the 33- and 100-ppm groups relative to air-exposed animals. Saillenfait *et al.* (1996) exposed rats to intermediate levels of 0, 400, 800, or 1200 ppm for 0.5 h once a day or to 0, 200, 400, 800, or 1200 ppm for 0.5 h 3 times a day during GD 6–15. They observed reduced fetal weight in Sprague-Dawley rats after repeated brief exposures (0.5 h 3 times per day) at 800 and 1200 ppm, but not with once a day exposures. There was no embryo lethality or teratogenicity following any exposure regimen. No data on GSH are available for any of the exposure regimens used in these studies.

Generoso and colleagues have evaluated the developmental effects of EtO exposure during the early postmating period. They exposed mice via inhalation at 1200 ppm per h for 1.5 hours at 1, 6, 9, and 24 h after mating (Generoso *et al.*, 1987), and observed late fetal deaths with exposure at 1 and 6 h after mating and resorptions at 9 h after mating. The types of malformations included hydrops and eye defects (54% of all anomalies), small size, cleft palate, and cardiac, abdominal wall, extremity, and tail defects (Rutledge and Generoso, 1989). In a follow-up study, Polifka *et al.* (1996) compared the effects of zygotic treatment with the effects of post-implantation exposure similar to that in the Laborde and Kimmel (1980) study. With post-implantation exposures, there were few external and visceral malformations, and the pattern of skeletal defects was somewhat different between the 2 groups. In particular, a high rate of major clefts of the sternum was seen in the zygote-treated group (58.5% at 3 h after treatment) versus 4.7% in the post-implantation group.

An interesting observation that requires further study is the relationship between exposure to EtO and the occurrence of micro- and anopthalmia, which was the most common malformation identified. This malformation is known to be more common in the inbred C57/BL6J strain of mice and to occur more often on the right side (Tyndall and Cook, 1990). Exposure to other teratogenic agents, including ethanol and retinoic acid (Cook and Sulik, 1988), has also been shown to increase the frequency of micropthalmia. The observations reported in this study are based on a subjective assessment of an examiner blind to the treatment group. To confirm the presence of an EtO-related effect, further quantitative measurements of the eyes are necessary and are currently being conducted with a reticle in the eyepiece of the dissecting microscope. These data, together with data on other skeletal defects will be reported in a subsequent paper.

Our study has raised a number of challenging statistical issues, especially related to experimental design. Because there is a fairly small region of the C × T surface where one can find developmental effects without maternal effects, running pilot studies to identify the optimal grid for the main study was necessary, but proved difficult and time-consuming. For compounds like EtO, where short, high exposures are expected to be the most toxic, we recommend identifying the highest tolerated concentration corresponding to the shortest exposure of interest and using this to define the highest multiple to be used in the experiment. There is substantial room for improved design strategies using modern techniques such as the Continual Reassessment method (O'Quigley *et al.*, 1990).

While our paper has focused largely on the dose-rate effects observed for developmental effects of ethylene oxide, our findings raise some interesting and important issues for risk assessment in general. Using methods developed by Williams and Scharfstein (1994), we calculated "effective dose contours" and illustrated how different benchmark doses could be computed for varied duration of exposure. It is also clear from our findings that use of Haber's-Law approach for risk assessment can easily under or overestimate the true risk, depending on the exposure duration involved. Further work is needed to determine how to appropriately incorporate dose-rate considerations into the risk-assessment process. Useful directions for research will include both basic toxicological investigations to identify the mechanisms that can explain dose rate effects, as well as statistical research to develop models that appropriately incorporate such findings into risk assessment.

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